Whole blood thrombin generation profiles of patients with cirrhosis explored with a near patient assay

Wan, Jun; Roberts, Lara N.; Hendrix, Wasiliki; Konings, Joke; Ow, Tsai-Wing; Rabinowich, Liane; Barbouti, Omar; De Laat, Bas; Arya, Roopen; Patel, Vishal C.

Published in:
Journal of Thrombosis and Haemostasis

DOI:
10.1111/jth.14751

Received: 14 November 2019 | Accepted: 27 January 2020

DOI: 10.1111/jth.14751

ORIGINAL ARTICLE

Whole blood thrombin generation profiles of patients with cirrhosis explored with a near patient assay

Jun Wan[1,2] | Lara N. Roberts[3] | Wasiliki Hendrix[1] | Joke Konings[1,2] | Tsai-Wing Ow[4] | Liane Rabinowich[4] | Omar Barbouti[4] | Bas de Laat[1,2] | Roopen Arya[3] | Vishal C. Patel[4,5,6] | Mark Roest[1,2] | Ton Lisman[7] | William Bernal[4,5]

[1]Synapse Research Institute, Maastricht, the Netherlands

[2]Cardiovascular Research Institute, Maastricht University, Maastricht, the Netherlands

[3]King's Thrombosis Centre, Department of Haematological Medicine, King's College Hospital, London, UK

[4]Institute of Liver Studies, King's College Hospital, London, UK

[5]School of Immunology and Microbial Sciences, Faculty of Life Sciences and Medicine, King's College London, London, UK

[6]Institute of Hepatology London, Foundation for Liver Research, London, UK

[7]Surgical Research Laboratory, Section of Hepatobiliary Surgery and Liver Transplantation, Department of Surgery, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

Correspondence
William Bernal, Institute of Liver Studies, King's College Hospital, London, UK.
Email: william.bernal@kcl.ac.uk

Ton Lisman, Department of Surgery, University Medical Center Groningen, Groningen, the Netherlands.
Email: j.a.lisman@umcg.nl

Abstract

Background and Aims: Patients with cirrhosis have a rebalanced hemostasis, often with normal or elevated thrombin-generating (TG) capacity in plasma. Whole blood (WB) TG allows faster determination and, importantly, includes the influence of all circulating blood cells. We aimed to study the TG profile of patients with cirrhosis in WB and in platelet poor plasma.

Methods: Thrombin-generating capacity in WB and plasma were assessed with a near-patient WB-TG assay and the calibrated automated thrombinography assay, respectively. TG assays were tested in presence and absence of thrombomodulin. Conventional coagulation tests were also performed.

Results: Thirty-four patients with cirrhosis and twenty-two controls were analyzed. Compared with controls, patients had substantially deranged results in conventional coagulation tests. Comparable WB-TG capacity (endogenous thrombin potential until peak, ETPp) but significantly lower peak thrombin were found in patients, and these results persisted when thrombomodulin was present. TG of the patients was more resistant to thrombomodulin than controls in both WB and plasma, although the inhibitory effect of thrombomodulin was drastically weaker in WB than in plasma. The peak of WB-TG in patients correlated moderately with their hematocrit and platelet count. Significant correlations were found between TG results in WB and plasma.

Conclusions: The WB-TG assay shows a normal to hypocoagulable state in patients with cirrhosis with a decreased anticoagulant activity of TM compared to plasma-TG. The clinical value of this assay needs further validation.

KEYWORDS

chronic liver disease, coagulation, thrombin generation, bleeding, thrombosis

Manuscript handled by: Robert Gosselin

Final decision: Robert Gosselin, 27 January 2020

1 | INTRODUCTION

Patients with liver dysfunction may acquire substantial alterations in their hemostatic system because the liver is responsible for the synthesis of the majority of hemostatic proteins. Conventional coagulation tests, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis, suggesting a generalized tendency toward bleeding. However, PT and APTT are only sensitive to changes in the procoagulant pathways, therefore reflecting only the abnormalities of the pro-coagulant proteins in cirrhosis, but providing little information about the anticoagulant pathways including antithrombin and proteins of the protein C pathway. Anticoagulant pathways may also be impaired in cirrhosis.[1-3] Clinical data show that cirrhotic patients are not "auto-anticoagulated," but rather have equal or even higher thrombotic risk than patients without liver disease.[2,4,5] These observations have led to the concept of rebalanced hemostasis to explain the complex coagulation profiles in patients with cirrhosis.[6-8] Although a hemostatic balance is reached in cirrhosis, this equilibrium is more fragile and may easily tip toward a hypo- or hypercoagulable state; however, traditional coagulation tests are not suitable to assess the functioning coagulation profiles in these patients.

Thrombin generation (TG) is a sophisticated laboratory test capable of assessing the complex interplay between pro- and anti-coagulant pathways, thus reflecting the function of the global hemostatic system. Modern TG assays, such as the calibrated automated thrombinography (CAT) assay, enable continuous TG measurement in plasma and have been shown to be predictive of thrombotic or bleeding risk in various coagulation disorders.[9,10] The onset, rate, and total capacity of TG in a given sample are represented by different parameters of a TG curve, ie, the lag time, peak thrombin, and endogenous thrombin potential (ETP), respectively.[9] By adding an activator of the anticoagulant protein C pathway, eg, thrombomodulin (TM) or Protac, a TG assay reflects the balance between all pro- and anticoagulant drivers. Studies using the TM-modified TG assays have revealed that the impaired procoagulant system is compensated for by the reduced anti-coagulant function in liver disease. This is reported to occur in both chronic liver disease and acute liver injury/failure.[6,7,11,12] These observations are in agreement with the clinical observations that there is no increased hemostasis-related bleeding risk for most patients.

Although the TM-modified TG assay was a crucial step forward in understanding the net effects of the multiple alterations in coagulation in cirrhosis, there are still several questions that remain to be answered. Standard TG assays are performed with platelet poor plasma (PPP) in the absence of blood cells, with synthetic phospholipids (PL) added to provide surfaces for the assembly of tenase and prothrombinase. These conditions may be different from physiological coagulation, which depends on the surfaces provided by activated platelets and most probably also erythrocytes.[13-16] During physiological coagulation there is a continuous interplay among coagulation initiation, platelet activation, propagation of coagulation, and erythrocyte suppletion.[13] In cirrhosis, anemia and thrombocytopenia are common and this could lead to reduced availability of procoagulant surfaces, which could potentially limit the rate and even capacity of TG. A study assessing TG in the platelet rich plasma (PRP) of cirrhotic patients demonstrated that platelet count is an important determinant of the ETP.[17] Although PRP-TG reflects the influence of platelets, it does not measure the additional impact of other blood cells. On the contrary, whole blood (WB)-TG includes the influence of all circulating blood cells on TG.[18] Another practical advantage of performing WB-TG over plasma TG is that no centrifugation is needed, avoiding potential pre-analytical variation and allowing faster results, which is crucial for point-of-care applications in critically ill or actively bleeding patients.[18]

We have previously developed a near patient WB-TG assay by using a miniaturized TG device and a microfluidic chip.[19] This assay was shown to be predictive of postoperative blood loss in patients undergoing elective cardiac surgery.[19] The short turnaround time and compact size of the near patient WB-TG assay system allows possible deployment outside a specialized laboratory environment. In this study, for the first time, we studied WB-TG profiles of a cohort of patients with cirrhosis by using the near patient assay.

Essentials

- Plasma thrombin generation (TG) tests support rebalanced hemostasis in patients with cirrhosis.
- Whole blood (WB)-TG profile in cirrhosis was tested by a near patient assay for the first time.
- Patients with cirrhosis have normal WB-TG capacity but with a lower rate.
- The anticoagulant effect of thrombomodulin was drastically weaker on WB-TG than on plasma-TG.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy controls

Patients with cirrhosis and healthy controls were recruited at King's College Hospital NHS Foundation Trust, London. The study was approved by the London (Camden & Islington) Research and Ethics Committee (REC number: 11/LO/0706) and the local Research and Development department at King's College Hospital NHS Foundation Trust. All participants provided written informed consent prior to study entry; where this was not possible, consultee permissions were obtained from their next of kin. Subjects were excluded from the analysis if they had acute liver failure or had received any anticoagulant or anti-platelet therapy.

2.2 | Blood collection and plasma preparation

Blood was collected into vacuum tubes (1 volume 0.109 mol/L trisodium citrate to 9 volumes blood; VACUETTE®, Greiner Bio-One). The blood was kept at room temperature and used within 4 hours after collection. PPP was prepared by double centrifugation of citrated WB at 2840 g for 10 minutes. Plasma was aliquoted and frozen at −80°C before analysis.

2.3 | WB-TG as measured with a modified near patient assay

WB-TG was measured on a previously described miniaturized TG device[19] and a newly modified microfluidic chip. The previous chip, which was made from methyl methacrylate-acrylonitrile-butadienestyrene (MABS), suffered from occasional fluid leakage.[19] We adopted a new design for the chip, which consisted of two pieces of polydimethylsiloxane (PDMS) with a porous matrix disc of 5 mm in diameter and 190 µm in thickness (grade 589/1; Whatman) in between. The two PDMS parts were prepared in house by mixing 10 parts of polymer with 1 part of curing agent (Sylgard 184, Dow Corning) and then solidified in an aluminum mold at 100°C for 90 minutes. After treatment in a plasma cleaner (PDC-32G-2, Harrick Plasma) for 15 minutes, the two PDMS parts were then assembled with filter paper in the center of the chamber between the two PDMS parts. Permanent bonds formed between the two PDMS parts, hence leakage was effectively prevented.

Citrated blood (15 µL) was mixed with 15 µL trigger solution containing 600 µmol/L fluorogenic thrombin substrate $(ZGGR)_2$-Rhodamine 110 (P_2Rho; Mercachem BV), 5 pmol/L recombinant human tissue factor (TF; Siemens Healthcare), and 33.4 mmol/L $CaCl_2$ in buffer containing 20 mmol/L HEPES, 140 mmol/L NaCl, and 5 g/L bovine serum albumin (Sigma) with a pH of 7.35. Fifteen microliters of the above mixture was immediately injected into the inlet of the microfluidic chip and the fluorescence measurement was started. An additional measurement was also done with soluble TM (U-protein Express) added into the TF-containing trigger solution. The final concentrations were 50% volume blood, 2.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho in the presence or absence of 150 nmol/L TM. This TM concentration was chosen based on dose-response experiments (Figure S1 in supporting information). For every blood sample an independent calibration experiment was performed in which 15 µL α_2-macroglobulin–thrombin complex (α_2M-T, ie, the calibrator, prepared as previously described[9]) was added to 30 µL of blood and substrate mixture; then 15 µL of the above mixture was injected into the chip and measured. The fluorescence signal was recorded with γ_{ex} = 470 nm and γ_{em} = 520 nm. The assay was performed at 37°C.

From the fluorescence data the WB-TG parameters were calculated using a Chapman-Richard growth (CRG)-based calculation method as previously described.[19] TG parameters selected for the analyses include the lagtime (the time from triggering coagulation until thrombin is detectable; min), time-to-peak (TTP, the time from initiation until the highest transient thrombin concentration; min), peak (the highest transient thrombin concentration; nmol/L) and endogenous thrombin potential until the thrombin peak (ETPp, ie, the area under the TG curve until the thrombin peak is reached; nmol × min/L). The TG parameters tested in the presence of TM are depicted as lagtime™$^+$, TTP™$^+$, peak™$^+$, and ETPp™$^+$, respectively. The interassay coefficient of variation (CV) of the assay was determined by testing six independent measurements per donor, the average inter-assay variation in five donors was 7.2%, 2.6%, 14.4%, and 13.7% for the lagtime, TTP, peak, and ETPp, respectively.

2.4 | PPP-TG measurement with the CAT assay

TG in PPP was measured with the CAT technique on a Fluoroskan Microplate Fluorometer (Thermolabsystems OY) as originally described.[9] The final concentration of TF was 5 PM with 4 µmol/L PL in the absence or presence of 10 nmol/L TM. The concentration of TM was chosen to inhibit TG by 50% in normal pooled plasma (NPP). TG parameters were calculated with Thrombinoscope software version 5.0, and TG parameters including the lagtime, TTP, peak, and ETP were chosen for further analyses.

In each run of TG measurement, NPP was also measured on the same plate. The ETP and peak values of the study subjects were normalized as the percentage of the ETP and peak of the NPP tested without TM in the same run, respectively. For better standardization and comparison, the PPP-TG parameters of samples measured in the presence of TM, including peak™$^+$ and ETP™$^+$, were also normalized as the percentage of the ETP and peak of the NPP tested without TM in the same run. The preparation of the NPP has been described previously.[20] Blood from 116 healthy adult volunteers who gave written consent and did not take any anticoagulant or antiplatelet drugs for at least 2 weeks before blood draw was collected at Maastricht University Medical Center. After an initial centrifugation step (2500 g, 5 minutes) plasma was pooled, followed by ultracentrifugation (100 000 *g*, 10 minutes). Aliquots of 500 µL were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

2.5 | Standard laboratory measurements

All coagulation assays were performed on the automated analyzer STA-R as previously described.[12] Reagents were obtained from Diagnostica Stago for all coagulation assays which were performed on the automated analyzer STA-R Evolution (Diagnostica Stago). APTT and international normalized ratio (INR) were measured by coagulation-based assays, with STA-Cephascreen and STA-Neoplastine, respectively. Clauss methodology was utilized to quantify fibrinogen with STA-Fibrinogen. Antithrombin activity was measured by a chromogenic substrate method, with STA-Stachrom

ATIII. Factor (F) II, VIII, and X were quantified with STA-deficient II, VIII, X immune-depleted plasmas using coagulation-based assays. Full blood cell count analysis was performed by a flow cytometry method and hemoglobin by a colorimetric method, with reagents from Bayer Diagnostics and the Advia 2120 automated hematology analyzer (Bayer Diagnostics).

2.6 | Statistics

Statistical analyses were performed with SPSS version 25 and graphs were generated using GraphPad Prism software version 6. Normality of the data was assessed using the Shapiro-Wilk test. Data are represented as median with interquartile range (IQR). Comparisons between two independent groups were performed with the Mann-Whitney test. Fisher's exact test was used to compare nominal data between groups. The Spearman test was used for the correlation analysis. A two-sided *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of patients and controls

In total 34 patients with cirrhosis and 22 healthy donors were included in the analyses. As shown in Table 1, the patient group was older ($P < .001$) but had similar gender distribution compared with controls. The etiology of cirrhosis included alcohol related liver disease (n = 17), non-alcoholic fatty liver disease (n = 7), viral hepatitis (n = 3), and primary sclerosing cholangitis (n = 3). In addition, drug induced liver injury, primary biliary cirrhosis, non-alcoholic steatohepatitis, and autoimmune disease were each found in one patient.

Four patients were classified as Child-Pugh class A, 18 patients were in class B, and 12 patients in class C. The median Model for End-Stage Liver Disease (MELD) score of the patients was 17.5 (IQR: 14.3-23.8). Twenty-six patients had acutely decompensated (AD) cirrhosis, with a CLIF-AD score of 51 (45-53). Six patients fulfilled criteria for chronic liver failure (ACLF), of which three patients had a CLIF-ACLF grade 2, three patients had grade 3.[21,22]

3.2 | Conventional coagulation tests and cell counts

Patients with cirrhosis had significantly distorted coagulation profiles compared to healthy controls, as shown in Table 1. Patients had significantly higher INR and longer APTT ($P < .001$ for both). Lower levels of both pro- and anticoagulant factors were found in the patients, such as FII, FX, and antithrombin, except FVIII, which was higher ($P < .001$ for all factors compared with control). Clauss fibrinogen was comparable between the patients and controls.

Platelet count and hematocrit of the patient group were noticeably lower than the local reference ranges. The median (IQR) values in the patients were 92.5 (59-159) × 10^9/L and 0.32 (0.27-0.37) for platelet count and hematocrit, respectively, whereas the respective local reference ranges were 150-450 × 10^9/L and 0.36-0.50. The white blood cell counts of the patients were within the local reference range. The cell counts of the controls were not measured.

3.3 | WB-TG profiles

Figure 1A shows representative WB-thrombograms of the patients and controls. As summarized in Table 2 and shown in Figure 1B, patients had significantly longer lagtime and TTP than controls ($P < .01$ for both; Figure 1B). The peak thrombin value was substantially lower in patients than in controls (median values were 118 and 179 nmol/L for patients and controls, respectively; $P < .001$). However, the ETPp was not significantly different between the patients and controls (230 [196-294] versus 247 [222-280] nmol × min/L, respectively; $P = .356$).

The addition of 150 nmol/L TM into the WB-TG assay substantially prolonged the lagtime in both patients and controls to a similar extent of 37% ($P = .926$). Conversely, the added TM only exhibited a weak inhibitory effect on ETPp. TM-induced inhibition on the ETPp was slightly lower in patients at 10.3 (2.0-19.9) % than in controls at 18.9 (12.7-27.5)% ($P = .048$). In the presence of TM, the ETPp™$^+$ was still similar between patients and controls ($P = .709$), whereas the peak™$^+$ remained lower in patients ($P < .001$; Table 2 and Figure 1C).

3.4 | PPP-TG profiles

We obtained TG data from 28 out of the 34 patients, because PPP was not collected from 5 patients, and no TG curve was obtained from 1 of the patients. When PPP-TG was measured in the absence of TM, comparable lagtime and TTP values were found in patients and controls. The peak and ETP were slightly but significantly lower (12.2% and 14% lower in median values, respectively) in patients (Table 3 and Figure 2A). When PPP-TG was tested in the presence of TM, the peak™$^+$ and ETP™$^+$ values of the patient plasma were significantly higher than the controls (35.8% and 58.7% higher in median values; $P = .009$ and $P < .001$, respectively; Table 3 and Figure 2B).

3.5 | Correlations of TG parameters with other factors

We explored a possible relation between TG parameters and other factors. Age of the study subjects was counterintuitively correlated with longer lagtime ($r = .435$) and TTP ($r = .405$), as well as lower peak ($r = -.301$) of WB-TG, but not with ETPp ($r = .021$, $P = .879$; Table 4).

TABLE 1 Characteristics and coagulation profiles of patients with cirrhosis and healthy controls

	Patients		Controls		
	n	Median (IQR)	n	Median (IQR)	*P* value
Age, years	34	58 (53.3–63)	22	32.5 (28–39.5)	<.001
Gender: female	34	10 (29.4%)	22	12 (54.5%)	.093
Etiology					
ALD		17 (50%)			
NAFLD		7 (20.6%)			
Viral hepatitis		3 (8.8%)			
PSC		3 (8.8%)			
Other		4 (11.8%)			
Laboratory tests					
Sodium, mmol/L	34	136 (134–138)			
Creatinine, μmol/L	34	79 (68–109)			
Albumin, g/L	34	31.5 (26–34)			
Bilirubin, μmol/L	34	43.5 (23.3–109)			
Child-Pugh classification					
A		4 (11.8%)			
B		18 (52.9%)			
C		12 (35.3%)			
MELD score	34	17.5 (14.3–23.8)			
Coagulation parameters					
Platelet, $x10^9$/L	34	92.5 (59–159)			
Hematocrit	34	0.32 (0.27–0.37)			
Hemoglobin, g/L	34	99.5 (88–118)			
White cell count, $x10^9$/L	34	4.65 (3.43–6.53)			
INR, ratio	25	1.47 (1.26–1.81)	20	1.02 (0.98–1.07)	<0.001
APTT, second	22	38.8 (35.2–43.3)	19	29.4 (28.6–31.5)	<.001
Fibrinogen, g/L	25	2.8 (1.72–4.15)	20	2.8 (2.5–3.12)	.802
FII, %	25	53 (42–72)	20	98 (88.3–105)	<.001
FVIII, %	26	242 (210–284)	20	118 (93–175)	<.001
FX, %	25	55 (47–67)	20	93.5 (87.8–108)	<.001
AT, %	27	57 (38–81.5)	20	104.5 (99–107.5)	<.001

Note: Values are median (interquartile range) or number (percentage).

Abbreviations: ALD, alcoholic liver disease; APTT, activated partial thromboplastin time; FII, factor II; FVIII, factor VIII; FX, factor X; INR, international normalized ratio; IQR, interquartile range; MELD, the Model for End-Stage Liver Disease; NAFLD, nonalcoholic fatty liver disease; PSC, primary sclerosing cholangitis

The PPP-TG parameters in the presence of TM also showed moderate to weak correlations with age, including the lagtime™$^+$ (r = .394) and ETP™$^+$ (r = .284).

Moderate to weak correlations were found between WB-TG parameters in the absence of TM with corresponding PPP-TG parameters in absence of TM, including the lagtime (r = .282), peak (r = .468), and ETP (r = .415; Table 4).

Positive correlations were found between the hematocrit of patients and their WB-TG parameters, including the peak (Spearman r = .434), ETPp (r = .417) (Table 4), and peak™$^+$ (r = .374). The peak value was also positively correlated with the platelet count of the patients (r = .559). The platelet count showed weaker correlation with the ETPp (r = .304, P = .085).

In addition, the platelet count was positively correlated with some parameters of the PPP-TG, including the lagtime (r = .529, P = .004), ETP (r = .45, P = .016), and lagtime™$^+$ (r = .528, P = .004). No significant correlation was found between hematocrit and PPP-TG parameters.

Cirrhosis severity, as represented by the Child-Pugh classification and MELD score, was correlated with longer lagtime and lower peak of WB-TG, both in the absence and presence of TM (P < .05 for all correlations). The above scores were also correlated with lower peak of PPP-TG (P < .05 for both correlations).

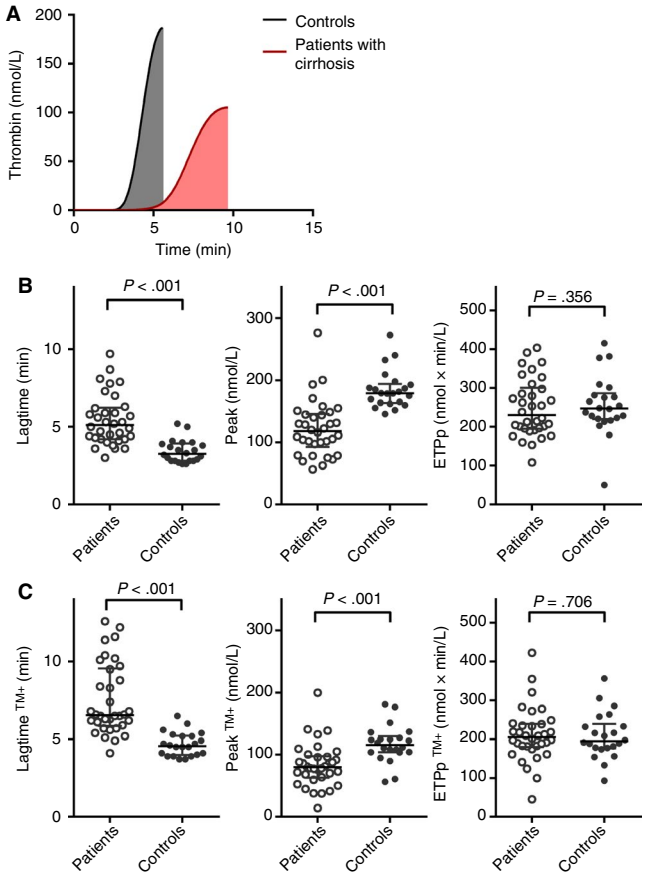

FIGURE 1 Whole blood thrombin generation (WB-TG) profiles of the study subjects. The whole blood thrombin generation profiles of patients with cirrhosis and healthy controls were studied with a near patient assay at 2.5 pmol/L tissue factor. A, representative curves of WB-TG of the patients (n = 34) and controls (n = 22) are shown. WB-TG parameters in the absence and presence of thrombomodulin (TM) are shown in (B) and (C), respectively. The Mann-Whitney test was used to compare between groups, *P* values are shown. Bars in the graph represent the median and interquartile range

4 | DISCUSSION

We studied the TG profiles of patients with cirrhosis in WB using a near patient WB-TG assay and in PPP with the standard CAT assay. WB-TG results showed normal TG capacity but lower TG rate in patients with cirrhosis, suggesting normal- to hypocoagulability; whereas TM-modified PPP-TG suggested hypercoagulability, with both higher TG capacity and rate found in cirrhosis. The WB-TG parameters showed moderate correlations with platelet count and hematocrit, as well as the standard PPP-TG parameters. We also surprisingly found that WB-TG is less sensitive to the anticoagulant function of TM than PPP-TG.

TG assays have been extensively used to study the coagulation profiles of patients with liver disease, and studies using TM-modified plasma TG assay have shown intact or elevated TG potential in cirrhosis, supporting the concept that these patients have re-balanced hemostasis.[6,23-27] However, until now all studies on TG profiles in these patients were performed in plasma samples, which has limitations, such as the requirement for a specialized laboratory environment, a longer turnaround time for results, and the inability to study the effect of most blood cells (except platelets) on TG. Until now there are no published data of WB-TG measurements in liver disease patients. Although whole blood viscoelastic assays have been tested in liver disease patients and shown additional value in reducing blood product administration, they do not assess the protein C pathway, and their ability to predict clinical outcome is still unclear.[28,29]

Using the near patient WB-TG assay, we observed that the endogenous thrombin generating potential (ETPp) values of patients with cirrhosis, regardless of the presence/absence of TM, were in the same range as in healthy controls, suggesting that these patients had normal thrombin-generating capacity. The standard PPP-TG results add to the growing literature that hypocoagulable state is suggested when TG is tested without activating the protein C pathway, whereas hypercoagulable state is found when the function of

TABLE 2 Whole blood thrombin generation profiles of patients and controls

	Patients (n = 34)	Controls (n = 22)	*P* value
WB-TG parameters in the absence of TM			
Lagtime (min)	5.1 (4.2–6.1)	3.3 (2.8–3.9)	<.001
TTP (min)	8.4 (7.3–9.9)	5.7 (5.1–6.8)	<.001
Peak (nmol/L)	118 (98–145)	179 (164–192)	<.001
ETPp (nmol × min/L)	230 (196–294)	247 (222–280)	.356
WB-TG parameters in the presence of TM			
Lagtime™$^+$ (min)	6.6 (6.1–9.3)	4.6 (4.1–5.2)	<.001
TTP™$^+$ (min)	10.9 (9.8–15)	8.1 (7.1–8.9)	<.001
Peak™$^+$ (nmol/L)	79.7 (65.1–96.2)	115 (104–127)	<.001
ETPp™$^+$ (nmol × min/L)	205 (181–238)	195 (177–233)	.709

Note: Values are median (interquartile range).

Abbreviations: ETPp, endogenous thrombin potential until the thrombin peak; TG, thrombin generation; TM, thrombomodulin; TTP, time to peak; WB, whole blood

TABLE 3 Plasma thrombin generation profiles in the absence and presence of thrombomodulin

	Patients (n = 28)	Controls (n = 22)	*P* value
PPP-TG parameters in the absence of TM			
Lagtime (min)	2.3 (2–3.3)	2.3 (2.3–2.6)	.627
TTP (min)	4.3 (3.8–5.3)	4.7 (4.3–5.1)	.082
Peak (% of NPP)	113 (89.1–128)	129 (115–137)	.019
ETP (% of NPP)	90.5 (82.3–101)	105 (96–121)	.001
PPP-TG parameters in the presence of TM			
Lagtime™$^{+}$ (min)	2.7 (2.3–3.6)	2.4 (2.3–2.7)	.043
TTP™$^{+}$ (min)	4.5 (4.1–5.3)	4.3 (4–4.5)	.102
Peak™$^{+}$ (% of NPP)	96.9 (74.6–108)	71.4 (52.1–83.1)	.009
ETP™$^{+}$ (% of NPP)	75.8 (67.4–84.5)	47.8 (38.1–58)	<.001

Note: Values are median (interquartile range); the peak and ETP values both in the absence and presence of TM were all normalized as the percentage of that of the NPP tested without TM in the sample run.

Abbreviations: ETP, endogenous thrombin potential; NPP, normal pooled plasma; PPP, platelet poor plasma; TG, thrombin generation; TM, thrombomodulin; TTP, time to peak

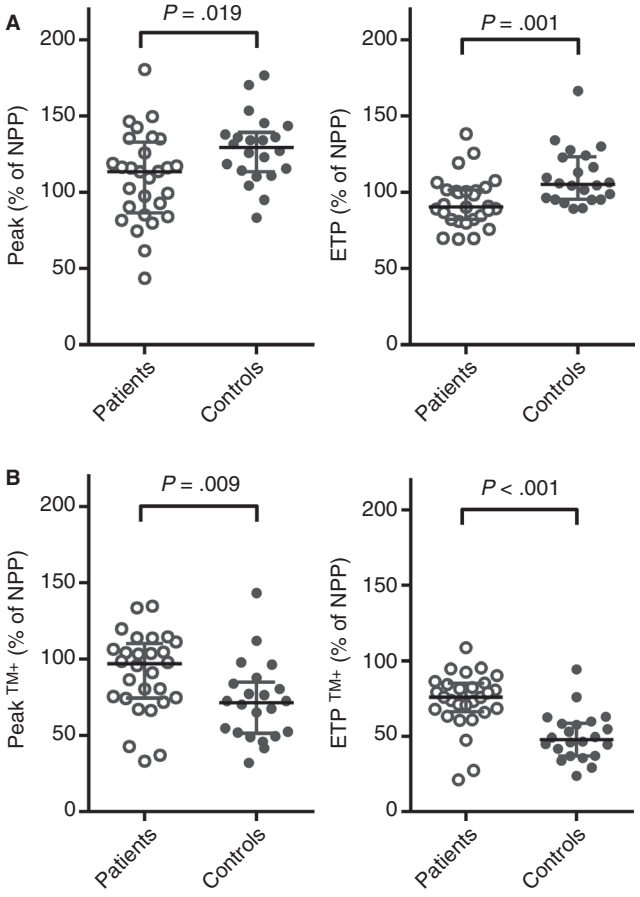

FIGURE 2 Plasma thrombin generation profiles of the study subjects. Platelet poor plasma of patients with cirrhosis (open circles) and healthy controls (filled dots) were tested with the calibrated automated thrombinography (CAT) assay at 5 pmol/L tissue factor, 4 μmol/L phospholipids in the absence or presence of 10 nmol/L thrombomodulin (TM). The peak thrombin and ETP values in the absence (A) and presence (B) of thrombomodulin (TM) are shown. All peak and ETP values both in the absence and presence of TM were normalized as the percentage of that of the normal pooled plasma (NPP) tested without TM in the sample run. The Mann-Whitney test was used to compare between groups

the anticoagulant protein C pathway is included in TG by adding TM into the test.[6,24-27,30] These results indicate that cirrhosis does not necessarily result in a hypocoagulable state as suggested by their lower procoagulant factor levels and prolonged PT and APTT, thus reinforcing that prophylactic administration of fresh-frozen plasma according to their PT/APTT is not required.[31,32]

Of note, although the TG capacity of cirrhotic patients is comparable to the controls, their peak thrombin level is discordantly lower, suggesting a hypocoagulable state. The ETP and peak are usually well correlated in PPP-TG, but their relation in WB has not been extensively explored. The above discordance might be due to a differential effect of low cell count on peak and ETPp. In our experience and in literature, the platelet count much more strongly affects the peak of PRP-TG than the ETP. In fact the ETP plateaued once the platelet count reached a certain level (approximately $100*10^9$ platelets/L) but the peak still increases with further ascending platelet counts.[33] The influence of erythrocyte count on WB-TG is less explored, but a recent study found that at a normal platelet concentration of $200*10^9$/L the peak of WB-TG was significantly augmented by increasing hematocrit from 0.2 to 0.45, whereas the ETP was largely unchanged.[34] Therefore, the lower counts of platelets and erythrocytes in cirrhotic patients might have induced differential impact on the peak and ETP of WB-TG, and caused the above-observed discordance. Platelets and erythrocytes are involved in TG via many mechanisms, including but not limited to their expression of procoagulant surface.[13,14,35-37] The activation/function of platelets in cirrhosis remains controversial.[38]

It has been reported that platelets are defective in aggregate formation, have a storage pool defect and defective transmembrane signaling in cirrhosis,[39] while other studies have reported that blood cells from cirrhotic patients have abnormally high levels of PS exposure and this may contribute to their hypercoagulable state.[40,41] Moreover, erythrocytes, as the most abundant cells in circulation, may also indirectly impact TG by augmenting platelet activation.[36,42] Overall the function of blood cells in TG of liver disease patients is still poorly characterized and this WB-TG assay has potential to improve insight into this topic.

Interestingly, WB-TG showed less sensitivity to the anticoagulant function of TM than PPP-TG. A high dose (150 nmol/L) of TM only induced weak inhibition on WB-TG, as the ETPp in patients and controls was only inhibited by 10.3% and 18.9%, respectively. Although the WB-TG of cirrhotic patients showed increased resistance to TM

TABLE 4 Correlations between WB-TG parameters and other factors

	WB-TG parameters					
	Lagtime	Peak	ETPp	Lagtime™⁺	Peak™⁺	ETPp™⁺
Age	0.435**	−0.301*	0.021	0.408**	−0.207	0.033
Platelet count	−0.147	0.559**	0.304	−0.346*	0.342	0.027
Hematocrit	−0.162	0.434*	0.417*	−0.289	0.374*	0.335
PPP-TG parameters						
Lagtime	0.282*	0.251	0.410**	0.219	0.156	0.079
Peak	−0.344*	0.468**	0.280*	−0.397**	0.460**	0.057
ETP	−0.295*	0.629**	0.415**	−0.432**	0.568**	0.121
Lagtime™⁺	0.449**	0.046	0.350*	0.397**	0.000	0.100
Peak™⁺	−0.008	−0.250	−0.178	0.042	0.006	0.009
ETP™⁺	0.241	−0.537**	−0.258	0.295*	−0.237	0.050

Note: Spearman correlation coefficients are shown.

Abbreviations: ETP, endogenous thrombin potential; ETPp, endogenous thrombin potential until thrombin peak; PPP, platelet poor plasma; TG, thrombin generation; TM, thrombomodulin; WB, whole blood

*$P < .05$.

**$P < .01$.

compared to controls, the difference was only on the border of statistical significance ($P = .048$). Conversely, a much lower dose of TM (10 nmol/L) exhibited a much stronger inhibitory effect on the ETP of PPP-TG and the effect was five-fold weaker in the patients than in controls. The TM resistance in cirrhosis is most probably due to their reduced protein C and protein S, combined with the elevated FVIII levels.[43-45] The high dose of TM, however, did substantially prolong the lagtime of WB-TG in both patients and controls, but this effect may be not via the anticoagulant function of the protein C pathway, but rather because TM prevented thrombin from activating platelets and consequently limited the availability of procoagulant phospholipids.[46]

This study has several limitations. First, the number of study subjects was relatively low and a heterogenous group of patients was included. Second, the age of the cirrhotic patients was higher than the healthy controls. However, the effect of age on thrombin generation in the general population is modest, and in patients with cirrhosis this effect is even smaller as cirrhosis-related distortions of the coagulation system outweigh the effects of age on plasma levels of coagulation factors.[26] This is further supported by our observation that older age was counterintuitively correlated with longer lagtime and lower peak of WB-TG. Furthermore, due to unknown reasons, WB-TG was more resistant to TM than PPP-TG despite being used at a high concentration. It is noteworthy that Lebreton et al[26] reported that plasmatic hypercoagulability of cirrhosis patients was detectable only when testing TG with the addition of TM but not activated protein C. Increased understanding of the function of TM in WB-TG is warranted to further optimize the TM-modified WB-TG assay for better evaluation of the protein C system. Future studies with larger sample size will be needed to explore the feasibility of this assay to assist coagulation management in liver disease patients.

ACKNOWLEDGMENTS

Jun Wan is supported by a scholarship (No.201606130068) from the China Scholarship Council, which were not involved in the study design, data interpretation, or the submission. The authors would like to thank all the participants of the study.

CONFLICTS OF INTEREST

All authors state that they have no conflictS of interest related to this study.

AUTHOR CONTRIBUTIONS

T. W. Ow, L. Rabinowich, and O. Barbouti recruited participants, and collected samples and patient data. J. Wan, W. Hendrix, and J. Konings performed thrombin generation experiments. R. Arya, W. Bernal, T. Lisman, L. N. Roberts, and M. Roest designed and supervised the study. V. C. Patel and B. de Laat assisted with analyses and writing of the paper. J. Wan, W. Bernal, T. Lisman, L. N. Roberts and M. Roest interpreted the data and wrote the manuscript. All authors approved the final version for submission.

ORCID

Jun Wan https://orcid.org/0000-0002-0948-8191
Lara N. Roberts https://orcid.org/0000-0003-3871-8491
Vishal C. Patel https://orcid.org/0000-0001-6616-3628
Ton Lisman https://orcid.org/0000-0002-3503-7140

REFERENCES

1. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *NEJM*. 2011;365:147-156.
2. Lisman T, Porte RJ. Pathogenesis, prevention, and management of bleeding and thrombosis in patients with liver diseases. *Res Pract Thromb Haemost*. 2017;1:150-161.

3. Tripodi A. Hemostasis in acute and chronic liver disease. *Semin Liver Dis*. 2017;37:28-32.
4. Northup PG, McMahon MM, Ruhl AP, et al. Coagulopathy does not fully protect hospitalized cirrhosis patients from peripheral venous thromboembolism. *Am J Gastroenterol*. 2006;101(7):1524-1528; quiz 680.
5. Sogaard KK, Horvath-Puho E, Gronbaek H, Jepsen P, Vilstrup H, Sorensen HT. Risk of venous thromboembolism in patients with liver disease: a nationwide population-based case-control study. *Am J Gastroenterol*. 2009;104:96-101.
6. Tripodi A, Salerno F, Chantarangkul V, et al. Evidence of normal thrombin generation in cirrhosis despite abnormal conventional coagulation tests. *Hepatology*. 2005;41:553-558.
7. Lisman T, Bakhtiari K, Adelmeijer J, Meijers JCM, Porte RJ, Stravitz RT. Intact thrombin generation and decreased fibrinolytic capacity in patients with acute liver injury or acute liver failure. *J Thromb Haemost*. 2012;10:1312-1319.
8. Lisman T, Porte RJ. Rebalanced hemostasis in patients with liver disease: evidence and clinical consequences. *Blood*. 2010;116:878-885.
9. Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003;33:4-15.
10. Tripodi A. Thrombin generation assay and its application in the clinical laboratory. *Clin Chem*. 2016;62:699-707.
11. Tripodi A, Fracanzani AL, Primignani M, et al. Procoagulant imbalance in patients with non-alcoholic fatty liver disease. *J Hepatol*. 2014;61:148-154.
12. Habib M, Roberts LN, Patel RK, Wendon J, Bernal W, Arya R. Evidence of rebalanced coagulation in acute liver injury and acute liver failure as measured by thrombin generation. *Liver Int*. 2014;34:672-678.
13. Hoffman M, Monroe DM. A cell-based model of hemostasis. *Thromb Haemost*. 2001;85:958-965.
14. Peyrou V, Lormeau JC, Herault JP, Gaich C, Pfliegger AM, Herbert JM. Contribution of erythrocytes to thrombin generation in whole blood. *Thromb Haemost*. 1999;81:400-406.
15. Horne MK 3rd, Cullinane AM, Merryman PK, Hoddeson EK. The effect of red blood cells on thrombin generation. *Br J Haematol*. 2006;133:403-408.
16. Whelihan MF, Zachary V, Orfeo T, Mann KG. Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation. *Blood*. 2012;120:3837-3845.
17. Tripodi A, Primignani M, Chantarangkul V, et al. Thrombin generation in patients with cirrhosis: the role of platelets. *Hepatology*. 2006;44:440-445.
18. Ninivaggi M, Apitz-Castro R, Dargaud Y, de Laat B, Hemker HC, Lindhout T. Whole-blood thrombin generation monitored with a calibrated automated thrombogram-based assay. *Clin Chem*. 2012;58:1252-1259.
19. Moorlag M, Schurgers E, Krishnamoorthy G, et al. Near patient thrombin generation in patients undergoing elective cardiac surgery. *JALM*. 2017;1:613-625.
20. Bloemen S, Zwaveling S, Douxfils J, Roest M, Kremers R, Mullier F. The anticoagulant effect of dabigatran is reflected in the lag time and time-to-peak, but not in the endogenous thrombin potential or peak, of thrombin generation. *Thromb Res*. 2018;171:160-166.
21. Jalan R, Pavesi M, Saliba F, et al. The CLIF consortium acute decompensation score (CLIF-C ADs) for prognosis of hospitalised cirrhotic patients without acute-on-chronic liver failure. *J Hepatol*. 2015;62:831-840.
22. Moreau R, Jalan R, Gines P, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology*. 2013;144:1426-1437, 37 e1-9.
23. Delahousse B, Labat-Debelleix V, Decalonne L, d'Alteroche L, Perarnau JM, Gruel Y. Comparative study of coagulation and thrombin generation in the portal and jugular plasma of patients with cirrhosis. *Thromb Haemost*. 2010;104:741-749.
24. Gatt A, Riddell A, Calvaruso V, Tuddenham EG, Makris M, Burroughs AK. Enhanced thrombin generation in patients with cirrhosis-induced coagulopathy. *J Thromb Haemost*. 2010;8:1994-2000.
25. Groeneveld D, Porte RJ, Lisman T. Thrombomodulin-modified thrombin generation testing detects a hypercoagulable state in patients with cirrhosis regardless of the exact experimental conditions. *Thromb Res*. 2014;134:753-756.
26. Lebreton A, Sinegre T, Pereira B, Lamblin G, Duron C, Abergel A. Plasma hypercoagulability in the presence of thrombomodulin but not of activated protein C in patients with cirrhosis. *J Gastroenterol Hepatol*. 2017;32:916-924.
27. Bos S, van den Boom B, Kamphuisen PW, et al. Haemostatic profiles are similar across all aetiologies of cirrhosis. *Thromb Haemost*. 2019;119:246-253.
28. Hugenholtz GCG, Lisman T, Stravitz RT. Thromboelastography does not predict outcome in different etiologies of cirrhosis. *Res Pract Thromb Haemost*. 2017;1:275-285.
29. Abeysundara L, Mallett SV, Clevenger B. Point-of-care testing in liver disease and liver surgery. *Semin Thromb Hemost*. 2017;43:407-415.
30. Russo FP, Zanetto A, Campello E, et al. Reversal of hypercoagulability in patients with HCV-related cirrhosis after treatment with direct-acting antivirals. *Liver Int*. 2018;38:2210-2218.
31. Lisman T, Caldwell SH, Burroughs AK, et al. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol*. 2010;53:362-371.
32. Rassi A, d'Amico E, Tripodi A, et al. Fresh frozen plasma transfusion in patients with cirrhosis and coagulopathy: effect on conventional coagulation tests and thrombomodulin-modified thrombin generation. *J Hepatol*. 2020;72:85-94.
33. Vanschoonbeek K, Feijge MAH, Van Kampen RJW, et al. Initiating and potentiating role of platelets in tissue factor-induced thrombin generation in the presence of plasma: subject-dependent variation in thrombogram characteristics. *J Thromb Haemost*. 2004;2:476-484.
34. Walton BL, Lehmann M, Skorczewski T, et al. Elevated hematocrit enhances platelet accumulation following vascular injury. *Blood*. 2017;129:2537-2546.
35. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev*. 2013;93:327-358.
36. Weisel JW, Litvinov RI. Red blood cells: the forgotten player in hemostasis and thrombosis. *J Thromb Haemost*. 2019;17:271-282.
37. Whelihan MF, Mann KG. The role of the red cell membrane in thrombin generation. *Thromb Res*. 2013;131:377-382.
38. Caldwell S, Lisman T. The cirrhotic platelet: shedding light on an enigma. *Hepatology*. 2017;65:407-410.
39. Lisman T. Hemostatic and non-hemostatic functions of platelets in patients with liver disease. In: Gresele P, Kleiman NS, Lopez JA, Page CP, eds. *Platelets in Thrombotic and Non-Thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics: an Update*. Cham, Switzerland: Springer International Publishing; 2017:1169-1181.
40. Lang E, Gatidis S, Freise NF, et al. Conjugated bilirubin triggers anemia by inducing erythrocyte death. *Hepatology*. 2015;61:275-284.
41. Wu X, Yao Z, Zhao L, et al. Phosphatidylserine on blood cells and endothelial cells contributes to the hypercoagulable state in cirrhosis. *Liver Int*. 2016;36:1800-1810.
42. Klatt C, Krüger I, Zey S, et al. Platelet-RBC interaction mediated by FasL/FasR induces procoagulant activity important for thrombosis. *J Clin Invest*. 2018;128:3906-3925.

43. Tripodi A, Primignani M, Lemma L, Chantarangkul V, Mannucci PM. Evidence that low protein C contributes to the procoagulant imbalance in cirrhosis. *J Hepatol*. 2013;59:265-270.
44. Sinegre T, Duron C, Lecompte T, et al. Increased factor VIII plays a significant role in plasma hypercoagulability phenotype of patients with cirrhosis. *J Thromb Haemost*. 2018;16:1132-1140.
45. Lisman T, Bos S, Intagliata NM. Mechanisms of enhanced thrombin-generating capacity in patients with cirrhosis. *J Thromb Haemost*. 2018;16:1128-1131.
46. Esmon NL, Carroll RC, Esmon CT. Thrombomodulin blocks the ability of thrombin to activate platelets. *J Biol Chem*. 1983;258:12238-12242.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wan J, Roberts LN, Hendrix W, et al. Whole blood thrombin generation profiles of patients with cirrhosis explored with a near patient assay. *J Thromb Haemost*. 2020;18:834–843. https://doi.org/10.1111/jth.14751